



Europäisches Patentamt

European Patent Office

Office européen des brevets

(11) Publication number:

0 097 907

A2

EUROPEAN PATENT APPLICATION

(21) Application number: 83106050.4

(22) Date of filing: 21.06.83

(51) Int. Cl.<sup>3</sup>: B 01 J 13/00

C 12 N 5/02, C 12 P 21/02

B 01 J 2/02

(30) Priority: 25.06.82 US 392218

(43) Date of publication of application:  
11.01.84 Bulletin 84/2

(84) Designated Contracting States:  
BE DE FR GB IT NL SE

(71) Applicant: FLOW GENERAL, INC.  
7655 Old Springhouse Road  
McLean Virginia 22102(US)

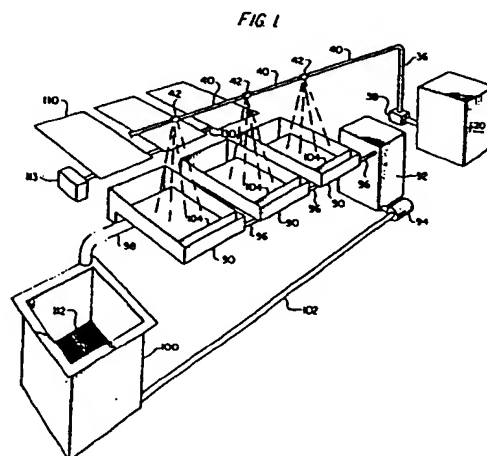
(72) Inventor: Haller, Wolfgang K.  
4620 N. Park Avenue Apt. 909E  
Chevy Chase, MD(US)

(74) Representative: Patentanwälte Grünecker, Dr.  
Kinkeldey, Dr. Stockmair, Dr. Schumann, Jakob, Dr.  
Bezold, Meister, Hilgers, Dr. Meyer-Plath  
Maximilianstrasse 58  
D-8000 München 22(DE)

(54) Cell culture microcarriers.

(57) Cell culture microcarriers made of water-insolubilized protein. The protein is substantially homogeneously distributed throughout the microcarrier. The cell culture microcarrier is made by forming droplets of an aqueous solution of a protein, suspending the droplets in a fluid, solidifying the suspended droplets into cell culture microcarriers and recovering the cell culture microcarriers. Preferably, prior to recovery, the cell culture microcarriers are hardened with a crosslinking agent, preferably glutaraldehyde and neutralized with a dilute solution of the protein. An apparatus for converting a water-based protein solution to microspheres. The apparatus has a heated, thermostatically controlled reservoir for the protein solution. The protein solution is supplied to a spray head. The protein solution is maintained at approximately at least the same temperature at the spray head as in the reservoir and a predetermined pressure is maintained on the protein solution at the spray head. A receptacle receives the droplets emitted by the spray head. The droplets are suspended and solidified into microspheres by flowing a low temperature, water immiscible suspension liquid across the receptacle and microspheres are collected. The cell culture microcarriers have been used to grow and

harvest anchorage dependent cells and anchorage dependent cell products, such as interferon. The cell culture microcarriers produce outstanding cell growth and possess superior subcultivation ability.



GRÜNECKER, KINKELDEY, STOCKMAIR &amp; PARTNER

PATENTANWALTE  
EUROPEAN PATENT ATTORNEYS

A. GRÜNECKER, DR. -ING.  
DR. H. KINKELDEY, DR. -ING.  
DR. W. STOCKMAIR, DR. -ING. & KATECH.  
DR. K. SCHUMANN, DR. -ING.  
P. H. JAKOB, DR. -ING.  
DR. G. BEZOLD, DR. -ING.  
W. MEISTER, DR. -ING.  
H. HILGERS, DR. -ING.  
DR. H. MEYER-PLATH, DR. -ING.

8000 MÜNCHEN 22  
MAXIMILIANSTRASSE 58

10. CELL CULTURE MICROCARRIERS

15 This invention relates to cell culture microcarriers.  
The invention also relates to methods of growing anchorage  
dependent cells and of producing both anchorage dependent  
cells and anchorage dependent cell products. The invention  
further relates to an apparatus for converting a water-  
20 based protein solution to microspheres.

The in vitro culture of cells is an important research  
tool, as well as an industrially useful process. Specifi-  
cally, it is important to produce cells and also to  
25 collect such useful cell products as viral vaccines,  
hormones and other substances, including interferons.

Cells that are not anchorage-dependent will grow freely  
when suspended in a suitable medium. For such cells,  
30 scaling up from the research laboratory to an industrial  
operation is not excessively difficult, particularly be-  
cause the cultures are essentially homogeneous. Conse-  
quently, the cells and the medium can be readily sampled  
for examination. The medium surrounding the cells can  
35 also be readily removed, replaced or otherwise modified.

In contrast, large scale culture of anchorage dependent  
cells has involved various difficulties. Traditionally,

1 such cells were grown on the inside surface of glass or  
plastic vessels, such as bottles and dishes. In large  
scale applications, these traditional growth techniques  
impose severe restrictions because of limited growth  
5 surfaces.

Moreover, such traditional techniques tend to waste  
growth medium because the growth-area to medium-volume  
ratio of containers such as bottles and dishes, cannot  
10 be increased above a certain value. Because the medium  
cannot be readily sampled, it is difficult to determine  
the properties of both the medium and the cells during  
growth. Further, the medium composition cannot be easily  
adjusted.

15 Improvements in the growth-area to volume ratio, as well  
as in the ability to adjust the sample medium, were pro-  
vided by techniques wherein cells were grown on the sur-  
face of stationary beds of solids, such as ceramic rings  
20 and glass tube segments. In such techniques, the medium  
is circulated through the stationary bed, allowing the  
medium to be monitored and adjusted at another point of  
the circulation loop. Unfortunately, such perfusive,  
stationary-supported cell culture growing methods do not  
25 provide cells that are accessible for sampling. Disad-  
vantageously, moreover, the stationary beds may become  
irreversibly clogged, leading to progressive cell death  
and further clogging as parts of the bed become inac-  
cessible to the life-supporting circulation medium. The  
30 problem of clogging becomes particularly severe if attempts  
are made to improve the growth-area to volume ratio by  
reducing the size of the solid bodies in the bed.

35 The practice of growing anchorage-dependent cells advanced  
remarkably with the introduction of the stirred micro-  
carrier technique, which is designed to extend all the  
advantages of homogeneous, suspended cell cultures to  
anchorage-dependent cells. In principle, cells are grown

- 1 on small carrier particles, also known as carrier bodies  
or cell culture microcarriers. These cell culture micro-  
carriers have the requisite shape, size and density to be  
suspended and fluidized in an agitated growth medium.
- 5 The cell culture microcarriers also have surface prop-  
erties suitable for supporting cell growth. See Van Wezel,  
216 Nature 64-65 (1967).

In particular, Van Wezel demonstrated that cells could  
10 attach to the positively charged surfaces of a commercially  
available dextran-based anion exchanger. The use of cell  
culture microcarriers was slowed, however, by the non-  
specific toxicity of the microcarriers to cells. Ex-  
amining the causes of this problem, Thilly and his co-  
15 workers found that a reduction in the number of positive  
charges on the ion exchange bead also reduced toxicity  
problems with positively charged dextran-based micro-  
carriers. See Levine et al., Somatic Cell Genetics 3,  
149 (1977); and Levine et al., U.S. Patents 4,189,534 and  
20 4,293,654. Other positively charged microcarrier beads,  
based upon polyacrylamide, are described by Monthony et  
al. in U.S. Patent 4,237,218.

Such positively charged ion-exchanger type beads, although  
25 successfully used in microcarriers, possess a significant  
disadvantage. Because of the electrostatic interaction  
between the normally negatively charged cells and the  
positively charged microcarriers, it is difficult to re-  
move the cells from the microcarriers without reducing  
30 the viability of a significant fraction of the cells.  
Consequently, the harvesting of cells or of cell-associated  
products and the subcultivation of cells onto additional  
microcarrier beads have both continued to be difficult  
and troublesome operations. Accordingly, workers in the  
35 art have sought improved cell culture microcarriers for  
obtaining improved methods of producing both anchorage  
dependent cells and anchorage dependent cell products.

1 The present invention overcomes the problems and disadvantages of the prior art by producing cell culture microcarriers which have surface structures conducive to cell attachment and growth of cells. The cell culture microcarriers of the present invention do not exhibit electrostatic interaction which interferes with cell removal for harvesting or subcultivation purposes. Moreover, it has been discovered that the cell culture microcarriers of the present invention are not toxic to cells. Therefore,  
10 an object of the present invention is to produce cell culture microcarriers having a surface structure conducive to cell attachment and growth of cells without electrostatic interferences with cell removal for harvesting or subcultivation purposes.

15

It is another object of the invention to produce cell culture microcarriers which are not toxic to cells.

It is a further object of the invention to produce a cell culture microcarrier in a single process without the necessity of grafting a material to the surface of the cell culture microcarriers.  
20

It is a further object of the invention to produce cell culture microcarriers at a reasonable cost by a method in which no grafting is needed, in which the cell culture microcarrier beads remain hot-water soluble throughout most of the collection process, in which cleaning is easy and hygienic, in which there are no problems of pot life associated with the material from which the cell culture microcarriers are made, in which microcarriers outside a certain size range can be recycled and in which cell culture microcarriers having a high size uniformity are obtained.  
30

35

It is a further object of the invention to provide cell culture microcarriers which can be used repeatedly in the growth of anchorage-dependent cells.

- 1 It is a further object of the invention to provide cell  
culture microcarriers from which attached anchorage-  
dependent cells can be separated by mild treatment with  
proteolytic agents, such as trypsin, or other agents, such  
5 as EDTA.

Additional objects and advantages of the invention will  
be set forth in part in the description which follows  
and in part will be obvious from the description or may  
10 be learned by practice of the invention. The objects and  
advantages of the invention may be realized and attained  
by means of the instrumentalities and combinations par-  
ticularly pointed out in the appended claims.

- 15 To achieve the objects and in accordance with the purpose  
of the invention, as embodied and broadly described herein,  
the cell culture microcarrier according to the present in-  
vention comprises a water-insolubilized protein, the pro-  
tein being substantially homogeneously distributed throug-  
20 out the microcarrier.

Further, to achieve the foregoing objects and in accordance  
with the purpose of the invention, as embodied and broadly  
described herein, the method for producing cell culture  
25 microcarriers according to the present invention comprises  
the steps of:

- (a) forming droplets of a solution of a protein;
- (b) suspending the droplets in a fluid;
- 30 (c) solidifying the suspended droplets into cell culture  
microcarriers; and
- (d) recovering the cell culture microcarriers.

Still further, to achieve the foregoing objects and in  
35 accordance with the purposes of the invention, as embodied  
and described herein, the method of growing anchorage  
dependent cells according to the present invention

1 comprises the steps of:

- (a) providing a suspension comprising cell culture microcarriers, an innoculum of the cells and a nutrient-  
5 containing growth medium, the microcarriers comprising a water-insolubilized protein, the protein being substantially homogeneously distributed throughout the microcarrier; and
- (b) maintaining the suspension under conditions conducive to cell growth.

10

Still further to achieve the foregoing objects and in accordance with the purpose of the invention, as embodied and broadly described herein, the method of producing anchorage dependent cells according to the invention comprises the steps of:

- (a) providing a suspension comprising cell culture microcarriers, an innoculum of the cells and a nutrient-containing growth medium, the cell microcarriers comprising  
20 a water-insolubilized protein, the protein being substantially homogeneously distributed throughout the microcarriers;
- (b) maintaining the suspension under conditions conducive to cell growth; and
- 25 (c) harvesting the cells.

Still further to achieve the foregoing objects and in accordance with the purposes of the invention, as embodied and broadly described herein, the method of producing anchorage-dependent cell products according to  
30 the present invention comprises the steps of:

- (a) forming a suspension, in a suitable cell culture medium, of cell culture microcarriers comprising a water-  
35 insolubilized protein, the protein being substantially homogeneously distributed throughout the microcarrier;
- (b) inoculating the culture with anchorage-dependent cells to form a cell culture;

- 1 (c) maintaining the cell culture under conditions conducive to production of cell products; and  
(d) harvesting the cell products.

5 Still further to achieve the foregoing objects and in accordance with the purpose of the invention, as embodied and broadly described herein, an apparatus for converting a water-based protein solution to microspheres according to the present invention comprises;

10

a heated, thermostatically controlled reservoir for the protein solution;

at least one spray head;

means for supplying the protein solution from the

15 reservoir to the spray head;

means for applying heat to the supplying means for maintaining the protein solution at approximately at least the same temperature as the protein solution in the reservoir;

20 means for maintaining a predetermined pressure on the protein solution at the spray head;

at least one receptacle positioned beneath the spray head for receiving the droplets emitted by the spray head;

25 means for flowing a low temperature, water immiscible suspension liquid across at least one receptacle for suspending and solidifying the received droplets into discrete microspheres in the suspension liquid; and  
means for collecting the microspheres from the suspension  
30 liquid.

Still further to achieve the foregoing objects and in accordance with the purpose of the invention, as embodied and broadly described herein, a method for converting a water-based protein solution into microspheres according to the present invention comprises the steps of:

35



1 spraying the protein solution as a liquid into a gaseous  
atmosphere to form discrete droplets;  
maintaining a temperature differential between the solution  
and the gaseous atmosphere wherein the gaseous atmos-  
5 phere is at a lower temperature than the temperature of  
the solution before spraying;  
flowing a cooled water-immiscible suspension liquid across  
the path of the fall of the discrete droplets to form  
microspheres suspended in the cooled suspension liquid;  
10 and  
collecting the microspheres from the suspension liquid.

The accompanying drawings, which are incorporated in and  
constitute a part of this Specification, illustrate em-  
15 bodiments of the invention and together with the descrip-  
tion, serve to explain the principles of the invention.

Fig. 1 shows a perspective view of a preferred apparatus  
for producing microspheres in accordance with the present  
20 invention.

Fig. 2 shows a cross-sectional view of a receptacle for  
receiving droplets as shown in Fig. 1.

Fig. 3 shows a cross-sectional view of a spray head,  
as shown in Fig. 1.

25 Fig. 4 shows a bottom view of a whirl plate, as shown  
in Fig. 3.

Fig. 5 shows a cross-sectional view of the whirl plate  
of Fig. 4.

Fig. 6 is a schematic diagram of the apparatus of Fig. 1  
30 in somewhat more detail.

Fig. 7 is a flow chart showing a method for producing  
cell culture microcarriers in accordance with the present  
invention.

Fig. 8 shows a bottom view of a nozzle, as shown in  
35 Fig. 3

Reference will now be made in detail to the presently  
preferred embodiments of the invention, examples of which

1 are illustrated in the accompanying drawings.

In accordance with the invention, cell culture micro-carriers comprise a water-insolubilized protein. Preferred proteins include collagen, casein, soybean protein,  
5 silk, keratin, egg proteins and blood proteins. More preferably, the protein is collagen.

The microcarrier may comprise the reaction product of a  
10 protein and a cross-linking agent. Preferably, the cross-linking agent is multifunctional. Suitable multifunctional cross-linking agents contain such functional groups as aldehydes, epoxies, haloalkyls, haloaryls, isothiocyanates, isocyanates, diazo, aryl nitrenes, azides,  
15 acylhalides, acylanhydrides, diolcarbonates, chloroformates, imidoesters, N-hydroxysuccinimidylesters, maleinimides, carboxydiimidazols, formylesters and halotriazines. Preferably, the multifunctional cross-linking agent is glutaraldehyde or 1,4-butanediol diglycidyl ether.  
20 Further preferred cross-linking agents include formaldehyde, epichlorohydrin, phosgene and aldehyde starch.

Preferably, the cell culture microcarriers have a diameter of about 50 to about 350 microns.

25 In accordance with the invention, droplets of an aqueous solution of a protein, such as described above, are formed. As is well-known, collagen may be liquified by heating and chemically degrading collagen to obtain an aqueous  
30 gelatin solution. The collagen may be obtained from pigskin via the well-known acid process. Alternatively, commercially available denatured collagen, such as gelatin type A, may be obtained and dissolved by heating in water.

35 To assure that the cell culture microcarriers obtained are uniform, well-known quality control tests such as viscosity, bloom rating, color, turbidity, isoelectric point and pH may be run on the collagen or other protein

- 1 used. In order for the collagen to have sufficient mechanical strength, the bloom rating of the collagen should be from approximately 150 to approximately 300.
- 5 The droplets of the solution of the protein may be formed in various ways. Preferred ways of forming the droplets include atomizing the solution from a pressure nozzle, atomizing the solution from a gas-propelled spray nozzle,
- 10 atomizing the solution from a rapidly spinning surface, atomizing the solution from an ultrasonically driven nozzle and emulsifying the solution. Suitable pressure nozzles, gas-propelled spray nozzles, rapidly spinning surfaces and ultrasonically driven nozzles are commercially available.
- 15 Methods for emulsifying solutions of proteins are well-known.

In accordance with the invention, the droplets of the solution of the protein are suspended in a fluid.

- 20 According to a preferred embodiment of the invention, the fluid, which may be a liquid medium or a gaseous medium, as explained in detail below, contains a solidification agent for the droplets. Preferred solidification agents include ammonia, an aldehyde, an epoxy compound and
- 25 epichlorohydrin. Preferably, the aldehyde is a multifunctional aldehyde. More preferably, the multifunctional aldehyde is glutaraldehyde which is readily available commercially.
- 30 Preferably, the epoxy compound is a multifunctional epoxy compound. More preferably, the multifunctional epoxy compound is 1,4 butanediol diglycidyl ether, which is commercially available.
- 35 A particularly suitable aldehyde solidification agent for the droplets is formaldehyde. As is well-known, formaldehyde is commercially available.

- 1 In accordance with the invention, the fluid may be either a gaseous medium or a liquid medium. Preferred gaseous media include air, hydrogen chloride, ammonia, formaldehyde, glutaraldehyde, or combinations of these specific gaseous compounds. Preferably, the gaseous medium is maintained at a temperature lower than the droplets. More preferably, the droplets of solution are at a temperature of about 50°C and the gaseous medium is at room temperature or lower.
- 10 The liquid medium is preferably non-flammable and either immiscible with the droplets of the solution or miscible with the droplets. Preferably, the temperature of the liquid medium is maintained in a range from 0 to 10°C. However, the temperature of the liquid medium should not be below the freezing point of the protein solution because freezing of the droplets mechanically disrupts the microcarrier structure.

Preferred liquid media which are immiscible with the droplets are slightly soluble in water and rapidly evaporate below 50°C without leaving a residue. Specifically preferred immiscible liquid media are the halogenated hydrocarbons. Preferred halogenated hydrocarbons include methylene chloride ( $\text{CH}_2\text{Cl}_2$ ), 1,1,1-trichloroethane, trichloroethylene and liquid trademarked Freon<sup>®</sup> products of DuPont de Nemours, E.I. & Co., which products contain fluorocarbons and/or chlorofluorocarbons, such as the well-known and readily available Freon<sup>®</sup> T.F.

- 30 Preferred liquid media miscible with the droplets include acids, bases and aldehydes. The acids may be inorganic or organic. Preferred inorganic acids include nitric acid, hydrochloric acid, phosphoric acid and sulfuric acid. Preferred organic acids include oxalic acid and formic acid.
- 35 Preferred bases include ammonia, alkali hydroxide and amines.

Aldehydes are also suitable liquid media which are miscible with the droplets. Preferred aldehydes are formaldehyde

1 and glutaraldehyde.

In accordance with the invention, the suspended droplets are solidified into cell culture microcarriers. When  
5 solidified, colliding microcarriers do not coalesce. Solidification may be accomplished by maintaining a temperature differential between the droplets of the solution of the protein and the fluid. Solidification may also result from a solidification agent for the protein  
10 which is present in the solution of the protein.

With respect to maintaining a temperature differential between the droplets of the solution of the protein and the fluid, the fluid may be hotter than the droplets of the  
15 solution or colder than the droplets of the solution. Further, the temperature of the fluid may be hotter than the temperature of the droplets of the solution and the solution may also contain an appropriate solidification agent, as described below. Further, to accomplish solid-  
20 ification, the fluid may be a gaseous medium, which may be maintained at a temperature lower than the droplets. The droplets, suspended in the gaseous medium may then be introduced into suspension in a liquid medium, which also may be maintained at a temperature lower than the droplets.  
25

With respect to accomplishing solidification by the presence in the droplet of a solidification agent, it is, of course, required that the solidification agent be sufficiently slow acting that the protein solution does not  
30 solidify prior to being suspended as droplets in the fluid. The solidification agent is also water-soluble.

Preferred water-soluble solidification agents include aldehyde, acid and base. As described above, the aldehyde  
35 may be a multifunctional aldehyde, such as glutaraldehyde, or a monofunctional aldehyde, such as formaldehyde. The acid may be inorganic, such as nitric acid, hydrochloric acid, phosphoric acid and sulfuric acid, or organic, such

1 as oxalic acid and formic acid. Preferred bases include ammonia, alkali hydroxide and amines.

In accordance with the invention, the cell culture micro-  
5 carriers are recovered using either conventional techniques or those described in detail hereafter. In some instances, after solidifying the microcarriers but prior to recovering the microcarriers, it may be desired to further harden the cell culture microcarriers. Preferably, the hardening is  
10 accomplished by a cross-linking agent.

To accomplish hardening, the microcarriers may be suspended in a liquid or gaseous medium containing the cross-linking agent. Preferred cross-linking agents are multifunctional,  
15 including multifunctional aldehydes and multifunctional epoxies. Preferably, the multifunctional aldehyde is glutaraldehyde.

More preferably, the glutaraldehyde is combined with a  
20 reducing agent, preferably sodium dithionite. Addition of a reducing agent, such as sodium dithionite, to the glutaraldehyde produces cell culture microcarriers which are nearly colorless. Other preferred cross-linking agents include formaldehyde and 1,4 butanediol diglycidyl ether.  
25

Preferably, the hardening effect of the cross-linking agent is further accelerated by the application of heat. More preferably, the hardening effect of the cross-linking agent is further accelerated by increasing the application of  
30 heat without liquifying the microcarriers. To achieve this, the microcarriers are subjected to a temperature ramp during which only occasional stirring is needed.

In a particularly preferred embodiment of the invention, the  
35 protein solution is a collagen solution and the cross-linking agent used for hardening is glutaraldehyde. Heat is initially applied to the cell culture microcarriers at 25°C for a period of time between about 1 and 25 hours, preferably,

1 15 to 20 hours. The heat is then increased to 70°C for a  
period of time between about 1 and 25 hours, preferably,  
6 to 10 hours, more preferably, 8 hours. Lastly, the  
heat is raised to 100°C and maintained for a period of time  
5 from about 1 to 25 hours, preferably, 15 to 20 hours, more  
preferably, 16 hours. It has been found that when heated  
over a temperature ramp, such as described above, the cell  
culture microcarriers harden sufficiently at the initial  
temperature, without liquifying, so that the temperature  
10 may subsequently be raised to accomplish further hardening,  
again without liquifying the microcarriers.

In those embodiments of the method of the present invention  
wherein the fluid contains a solidification agent, a  
15 solidification agent is present in the solution of the  
protein or hardening is accomplished by a cross-linking  
agent, it has been found to be highly preferable, prior to  
the step of recovering the cell culture microcarriers, to  
neutralize the excess solidification or cross-linking  
20 agent from the cell culture microcarrier.

Neutralization is effected by treating the microcarrier  
with a dilute solution of the same protein used to form  
the droplets. Of course, the solution must be sufficiently  
25 dilute to avoid solidification of the protein. For a  
particular protein, one skilled in the art will routinely  
determine, without undue experimentation, how dilute a  
neutralizing solution should be to accomplish effective  
neutralization. In the case wherein collagen solution is  
30 used, a dilute collagen solution of about 0.5% by weight  
may be used to effect neutralization.

In addition to neutralizing, it may also be advantageous to  
exhaustively wash the cell culture microcarriers, prior to  
35 the step of recovering, with saline: Preferably, the  
microcarriers are washed at least once with saline before  
and after neutralization.

1 Further, it may be desired, either before or after the step  
of recovering the cell culture microcarriers, to sterilize  
the cell culture microcarriers. Techniques for steriliz-  
ing cell culture microcarriers, such as steam sterilization,  
5 are well-known.

The cell culture microcarriers of the present invention may  
be used to grow anchorage-dependent cells. Extensive  
information on cell culutres is readily available. See  
10 e.g., Tissue Culture, Methods and Applications (Ed. Kruse  
and Patterson) Academic Press, New York, 1973. Moreover,  
a great deal of information relating to cell culture micro-  
carriers is described in Volumes 46 (1980) and 50 (1982) of  
Developments in Biological Standardization. The disclosures  
15 of all these references are specifically incorporated by  
reference herein.

The anchorage-dependent cells and/or the anchorage dependent  
cell products which are grown on the cell culture micro-  
20 carriers of the present invention may be harvested by any  
of the conventional, well-known techniques.

For reasons already explained, the cell culture micro-  
carriers of the present invention greatly facilitate sub-  
25 cultivation. Conventional techniques for subcultivation  
in tissue cultures are well-known. Subcultivation of  
microcarrier cultures has heretofore been difficult to  
achieve because, as explained above, it has been difficult  
to remove the cells from the carriers and thereafter achieve  
30 effective cell growth. A preferred technique for subcultivat-  
ing in accordance with the present invention involves  
separating the cell growth from the microcarriers by tryps-  
inization, followed by introducing additional cell culture  
microcarriers. Alternatively, the cell growth is trypsinized  
35 to separate the cells from the microcarriers. The cells  
are then removed, for example by screening, from the growth  
medium containing the microcarriers and introduced into a  
new growth medium. To this new medium, new cell culture



1 microcarriers are added, for example, in double the number  
of previous microcarriers.

As illustrated in the drawings, the apparatus of the  
invention for converting water-based protein solution,  
5 as defined above, to microspheres, such as cell culture  
microcarriers, includes a heated thermostatically controlled  
reservoir for the protein solution. As shown in Figures 1  
and 6, the reservoir 20 may be a box-like receptacle having  
means for maintaining the protein solution 22 at a stable  
10 temperature, such as 50°C.

Preferably, the basic supply of protein solution 22 is kept  
in a first container 24 within the reservoir 20, the  
15 container being positioned in a bath 26, containing a heat-  
ing fluid such as glycerin or water, for maintaining the  
stable temperature. As shown schematically in Figure 6, a  
heater 28, controlled by a thermostat 30, supplies heat to  
the bath 26.

20 For purposes of rinsing and priming the apparatus, as  
described hereinafter, the reservoir 20 also includes a  
second container 32 for holding water 34, the second con-  
tainer also being positioned in the bath 26, for being  
25 maintained at the same temperature as the protein solution  
22.

In accordance with the invention, the apparatus includes  
means for supplying the protein solution from the reservoir  
30 through at least a substantially horizontal pipe. Preferably,  
the supplying means includes a loop from and back to the  
reservoir, the loop including an elongated, substantially  
horizontal pipe. As embodied herein, the supplying means  
includes a main liquid communication channel 36 forming a  
35 loop including a high pressure pump 38 for circulating the  
protein solution from the first container 24 through the  
loop and at least part of the solution back to the first  
container. For simplicity of illustration, the communication

1 channel 36 in Fig. 1 does not show the complete loop. The  
complete loop is depicted symbolically, however, in Figure 6.

Preferably, the terminal portion of the communication channel  
5 36 should include flexible members 39a, b, such as hoses.  
Either or both of the flexible members 39a, b may be  
selectively moved between the first and second containers 22,  
34 for priming or flushing the system with warm water. The  
apparatus preferably also includes a drain 41, shown  
10 symbolically in Figure 6, through which the system can be  
drained if desired. The drain would, of course, be  
positioned for accessibility by the flexible member 39a, b.

Preferably also, the container 24 includes a screen or  
15 filter 43 for receiving the input end of the communication  
channel 36 through the flexible member 39a to minimize the  
possibility of foreign materials entering the communication  
channel.

20 The communication channel includes an elongated, substantially  
horizontal pipe 40, having spaced therealong a plurality of  
spray heads 42. Only three spray heads 42 are illustrated,  
but it will be understood that any convenient number of  
spray heads may be utilized. Advantageously, the spray heads  
25 42 may be placed in series, as is shown in Figs. 1 and 6.

If the high pressure pump 38 is a piston-driven pump, the  
communication channel 36 should include a buffer 44 and  
restrictor 46 for attenuating any pulses developed in the  
30 circulating protein solution by the pump. Preferably, the  
buffer 44 and restrictor 46, if utilized, should be located  
in the communication channel 36 immediately downstream from  
the pump 38 and certainly between the pump and the spray  
heads 42. The restrictor 46 may include a filter, if  
35 desired (not shown).

The spray heads 42 of the invention are preferably structured  
as illustrated schematically in Figure 1 and shown in

1 detail in Figs. 3 to 6. As to each spray head 42, the  
heated protein solution enters the spray head 42 through  
an inlet port 48 into a cavity 50 of the body 52 of the  
spray head. The protein solution is forced downward along  
5 a barrier 54 into a spray sump 56 and outward through a  
nozzle 58. Preferably, the nozzle 58 is formed as a disc  
having a flat upper surface and a circular whirl-plate 60  
is mounted in the spray head 42 between the sump 56 and  
nozzle disc 58.

10

As shown more particularly in Figs. 4 and 5, the circular  
whirl-plate 60 is fashioned with at least a pair of canals  
62 leading from the sump 56 to a central circular hollow  
area 64 in the bottom of the whirl-plate and flush against  
15 the nozzle disc 58. The canals 62 are symmetrically spaced  
about the center of the whirl-plate 60 and intersect the  
hollow area 64 tangentially. As shown in Figure 4, the  
canals 62 intersect the area 64 on opposite sides of the  
area. The canals 62 may be L-shaped and formed of a  
20 vertical portion 63, such as a hole, and a horizontal portion  
65. The horizontal portion 65 may be a groove in the bottom  
of the whirl-plate 60. This structure induces rotary fluid  
motion in the hollow area as the protein jet departs the  
nozzle through a short exit 66. The rotary fluid motion  
25 imparted to the jet induces jet breakup at a lower nozzle  
velocity than would be required without such motion.

Preferably, the sump 56 is provided with a transverse screen  
68 of sufficient fineness to prevent materials from clogg-  
30 ing the canals 62 of the exit 66. The screen 68 may be  
clamped between an annular space 70 and an annular gasket  
72, the spacer and gasket being locked between the body 52  
of the spray head and a threaded nut 74.

35 If desired, the nozzle disc 58 may have its lower surface  
machined to form a depression 76 to give strength and  
rigidity to the disc and to provide outlet space for the  
exiting spray cone 78 without impingement of the outer

1 surface of the disc.

Also, if desired, the exit 66 may be made noncircular and elongated, instead of circular, for shaping the spray cone 5 78 as will be described in more detail hereinafter.

Preferably also, the canals 62 are about 0.040" in diameter and the exit 66 in the nozzle disc 58 is about 0.012" long and 0.005" in diameter. This structure of the spray head 10 induces jet instability and breakup of the protein jet into droplets. In addition, it allows droplet formation at relatively low spray pressures and high solution viscosity.

The loop including the diameters of the communication channel 15 36 and the horizontal pipe 40, together with internal dimensions of the spray heads 42 and the diameters of the canals 62 and exit 66 are such that only a small proportion of the protein solution circulating through the loop passes through the spray heads during one cycle of the protein 20 solution. This design contributes to the stability of temperatures of the protein solution in the spray heads, discussed in more detail hereinafter.

The protein solution not passing through the nozzle 58 is 25 forced upwardly through the cavity 50 on the opposite side of the barrier 54 and outward through the outlet port 79 into the horizontal pipe 40.

Within the barrier wall 54 is formed a thermal sensor well 30 80 in which is placed any convenient device for monitoring the temperature of the spray head 42. Such a device may be a thermometer for optical monitoring, or a more sophisticated device for registering the temperature in a computerized control.

35

It is of great importance that the temperature of the spray head remain stable since temperature affects viscosity of the protein solution. Viscosity is an important parameter

1 affecting the spray rate delivered by the spray heads and  
the size distribution of the droplets in the spray. Most  
preferably, when more than one spray head in series is  
used, the temperature of the protein solution at the inlet  
5 port 48 is the same as the temperature of the protein  
solution at the outlet port 79.

The apparatus for the invention includes means for applying  
heat to the pipe for maintaining the protein solution in the  
10 spray heads at approximately at least the same temperature  
as the protein solution in the reservoir. Since excessive  
heat degrades the protein, it is preferred to store the  
protein solution at a lower temperature in reservoir 20,  
but still sufficiently high to maintain a pumpable viscosity,  
15 and to heat the protein solution to a higher temperature as  
the solution approaches a spray head. As embodied herein,  
the means for applying heat includes heating jackets 84  
wrapped around the elongated horizontal pipe 40 in selected  
positions. Preferably, the heating jackets 84 are positioned  
20 upstream of the spray heads.

If desired, heating jackets may also be placed around the  
communication channel 36 at points remote from the spray  
heads 42 to maintain the temperature sufficiently high that  
25 the solution is maintained at a pumpable viscosity. By use  
of the heating jackets, predetermined temperatures can be  
maintained throughout the loop.

30 In accordance with the invention, the apparatus for convert-  
ing a water-based protein solution to microspheres includes  
means for maintaining a predetermined pressure on the  
protein solution at the spray heads. As embodied herein,  
the pressure-maintaining means includes an adjustable  
throttle valve 86 for adjusting the pressure of the protein  
35 solution on the spray heads. In addition, the apparatus  
includes at least one pressure gauge 88 for monitoring the  
pressure on the loop.

1 The throttle valve 86 and pressure gauge 88 shown schematic-  
ally in Fig. 6 may be manually operated and optically  
observed respectively, or may be more sophisticated devices  
for complete control of the apparatus of the invention.

5

The apparatus of the invention also includes at least one  
receptable positioned beneath a spray head for receiving the  
droplets emitted by the spray head. Preferably, the number  
of receptables is equal to the number of spray heads.

10 Preferably, as stated above, there are a plurality of spray  
heads 42 spaced along the horizontal pipe 40 and a like  
plurality of receptacles 90 for individually receiving the  
droplets in the cones 78, emitted by the spray heads.

15 In accordance with the invention, the apparatus also  
includes means for flowing a low temperature, water immisc-  
ible suspension liquid (fluid liquid medium as described  
above) across the receptacle for suspending and solidifying  
the received droplets into discrete microspheres in the

20 liquid suspension. As embodied herein, the flowing means  
includes a reservoir 92 for containing a supply of the  
suspension liquid and a pump 94. The pump 94 drives the  
suspension liquid from the reservoir 92 through a feed pipe  
96 having an input at one end of each receptacle 90. The

25 suspension liquid flows downward along the length of the  
receptacle 90 and into a common drain 98 which empties into  
a collector 100. A suction pipe 102 pulls the suspension  
liquid from the bottom of the collector 100 back to the  
reservoir 92 under the power of the pump 94 completing the  
30 loop.

Preferably, as described above, the suspension liquid  
(liquid fluid medium) is a halogenated hydrocarbon selected  
from the group consisting of  $\text{CH}_2\text{Cl}_2$ , 1-1-1 trichloroethane,  
35 trichloroethylene and Freon<sup>®</sup> T.F.

The reservoir 92 includes means such as a cooling unit for  
maintaining the low temperature suspension liquid at a

1 temperature of approximately 0° to 10°C, but not below the  
freezing point of the protein solution. To maintain the  
low temperature of the suspension liquid, the whole apparatus  
may be placed in a cold room or the suspension liquid may be  
5 passed through a conventional cooler/heat-exchanger, which  
may be placed in the reservoir 92.

The receptacles 90 are rectangular in shape. Each of the  
receptacles 90 includes means for developing a pressure  
10 head of the suspension liquid across the input section of  
the receptacle for increasing the speed of flow of the  
liquid across the receptacle. As embodied herein, the  
pressure head-developing means is a barrier 104 across the  
input end of the receptacle and spaced slightly above the  
15 bottom of the receptacle. A pressure head of the suspension  
liquid therefore builds up behind the barrier 104, as shown  
in Fig. 2, forcing the liquid 106 with increased velocity  
out from under the barrier 104 and along the bottom of the  
receptacle 90.

20

Each receptacle 90 also includes a depressed area 106 at  
the end opposite the barrier for producing a cascade  
across the rectangular receptacle 90. The suspension liquid  
then flows out of each receptacle 90 through an outlet 108  
25 into the common drain 98 and into the collector 100.

The receptacles 90 are preferably dimensioned and positioned  
for receiving the droplets emanating from the individual  
spray heads 42 only in the flow of the suspension liquid in  
30 the receptacles. As indicated above, the exit apertures 66  
of the nozzles 58 may, as shown in Fig. 8, be made non-circular  
and elongated, if desired, to tend to shape the cones 78,  
which helps to confine each cone in an individual receptacle  
90.

35

Closable gate means 110, as shown in Fig. 1, may be provided  
for directly receiving the discharge from one or more spray  
heads. By such means the spray cone for one or more

1 receptacles may be prevented from entering the receptacles  
without shutting down the whole apparatus while individual  
receptacles are being repaired or replaced and also during  
priming, shutdown and cleaning operation. Closable gate  
5 drive means 113, including drive shaft 130, are used to move  
the closable gate, such as shutters, from the open position,  
wherein the spray cone discharges into the receptacles, to  
the closed position, wherein at least one spray cone is  
prevented from entering the receptacles.

10

As the droplets in the spray cones fall into the low temperature flow of the liquid suspension, the water-based protein solution is not miscible in the liquid solution. Consequently, the droplets remain integral and solidify in  
15 the cold suspension liquid. It is important that the suspension liquid move sufficiently fast to maintain a low steady state concentration of spheres in the liquid surface to prevent still-liquid droplets from impinging on droplets swimming on the surface. Such impinging produces twins and  
20 aggregates which are undesirable.

The apparatus of the invention for converting a water-based protein solution to microspheres also includes means for separating the microspheres developed in the receptacles  
25 from the liquid suspension. As stated above, the suspension liquid containing the microspheres flows into a common drain 98 as a common discharge. The collector 100 receives the common discharge and the microspheres are filtered therefrom, preferably by screen 112 extending across the  
30 collector. Preferably, the screen 112 has a mesh size of about 500  $\mu\text{m}$  and may be removably mounted in the collector 100 by any convenient, known structure.

The invention also includes the method for converting a  
35 water-based protein into microspheres comprising spraying the protein solution, described above, as a liquid into a gaseous atmosphere (fluid gaseous medium, as described above) to form discrete droplets, maintaining a temperature differential



1 between the solution and the gaseous atmosphere wherein the  
gaseous atmosphere is at a lower temperature than the  
temperature of the solution before spraying, flowing a cooled  
water immiscible suspension liquid (fluid liquid medium, as  
5 described above) across the path of fall of the discrete  
droplets to form microspheres suspended in the cooled sus-  
pension liquid, and collecting the microspheres from the  
suspension liquid.

10 The detailed steps of the invention parallel the details of  
the structure and the functioning of the structure as de-  
scribed hereinbefore.

The following examples are designed to elucidate the teach-  
15 ings of the present invention, and in no way limit the scope  
of the invention. Various other modifications and equivalents  
of the examples will readily suggest themselves to those of  
ordinary skill in the art, particularly after the issuance  
of this patent, without departing from the spirit or scope of  
20 the present invention.

The process described below generally conforms to the schem-  
atic diagram shown in Fig. 7.

25 1. Preparation of solution of denatured, degraded animal  
collagen.

In a 5 liter container; 440 grams of dry, granular acid-pro-  
cessed (Type A) gelatin (Bloom rating of 250) are dissolved  
30 in 3960 ml of distilled water. The gelatine granules are  
stirred into cold water. Stirring is continued while the  
temperature is raised to 60°C. Stirring is further continued  
until a homogeneous viscous solution is obtained. Stirring  
is then briefly interrupted until bubbles clear out, where-  
35 upon stirring is resumed slowly to assure homogeneity.

2. Start-up of protein spray loop.

1 With reference to Fig. 6, containers 32 and 24 are respectively filled with warm water 34 and a warm protein solution 22, such as the denatured, degraded animal collagen solution, described above, which solution may also contain a slow acting crosslinker, as described above. Thermostat 30 is set for 50°C. The hose flexible members 39a and 39b are placed in hot water container 32. Throttle valve 86 is completely opened. The spray shutter closable gate means 110 are completely closed. Pump 38 is started and hot water is  
10 circulated until temperature indicators show steady temperature. The appropriate power setting for heating jackets will either have been determined in previous runs with protein solution or will have to be adjusted in the first run. Hose flexible member 39b is moved to drain 41. Hose flexible  
15 member 39a is moved to protein solution 22. As soon as the protein solution has displaced the water in the loop, hose 39b is moved from the drain to the protein container 24. If not already set properly, controls to heating jackets 84 are adjusted until indicators on spray heads 78 show 60°C and  
20 the indicator on return 120 shows 50°C. The throttle is now closed until the pressure gauge 88 reaches 170 psi. Of course, spray heads having larger spray patterns will require other pressures, as will be obvious to those of ordinary skill in the art. If necessary, the power to the heating  
25 jackets is re-adjusted. These power levels should be noted and kept for later runs. At this stage, sprays of hot protein are discharged onto the spray shutter closable gate means 110. In the described design of Fig. 6 wherein ten spray heads are present, (Fig. 6 shows only three spray  
30 heads) the consumption rate of protein solution is 300 ml per minute. An initial amount of approximately 4 liter protein solution, such as the approximately 4 liter collagen solution prepared above, would therefore be consumed in 10 to 15 minutes.

35

### 3. Start-up of suspension liquid circulation loop.

With reference to Fig. 1, the pump 94 is started and a flow

1 of  $\text{CH}_2\text{Cl}_2$  suspension liquid is adjusted to approximately  
30 gallons per minute. Valves (not shown in Fig. 1) between  
the feed pipe 96 and the tray receptacles 90 are once per-  
manently adjusted to give equal flow rate to all trays. At  
5 this circulation rate, a 0.25" deep layer of liquid in each  
of ten 12" wide tray receptacles (only three tray receptacles  
are shown in Fig. 1) will produce a linear flow rate of  
10 cm/sec. A heat-exchanger/cooler (not shown in Fig. 1) is  
adjusted to give a suspension-liquid temperature between  
10 0.1°C and 10°C in the trays. In a variation, the suspension  
liquid may contain a diffusible crosslinking agent, as des-  
cribed above. As a further variation, a volatile, diffusible  
crosslinking agent, as described above, may be contacted  
with the spray cones prior to contract of the spray droplets  
15 with the suspension liquid.

#### 4. Production of sphere suspension in $\text{CH}_2\text{Cl}_2$ .

The spray shutter closable gate means 110 are now opened  
20 and droplets of protein solution impinge on the suspension  
liquid and solidify to discret spheres. The spheres of  
solidified protein are collected by straining on the screen  
112 in collector tank 100. The spheres should have a size  
distribution of between 250 and 330  $\mu\text{m}$ . The  $\text{CH}_2\text{Cl}_2$  which  
25 passes through the screen may be cooled and recycled to the  
liquid suspension reservoir. After nearly all the protein  
solution has been consumed, the spray shutters are closed.  
Pump 94 and the cooling means in the reservoir 92 are shut  
off. The return hose 39b is moved to drain 41 and the  
30 aspirator hose 39a is moved to the hot water container 32.  
When the circulation loop has been completely flushed with  
water, all power is shut off.

#### 5. Processing of protein sphere suspension in saline 35 solution.

(a)  $\text{CH}_2\text{Cl}_2$  removal.

1 Collection tank 100 now contains a cohesive cake of protein  
microspheres (bead cake) on a coarse screen 112 of 500  $\mu\text{m}$   
mesh size. The screen with the cake is now immersed into a  
container with saline and the cake is pressed through the  
5 screen into the container resulting in a mashed cake in  
saline.

The screen is now inserted into another empty container and  
the suspension of spheres is poured into the screen without  
10 application of pressure. The drained liquid contains some  
 $\text{CH}_2\text{Cl}_2$  and may be discarded. The cake on the screen is now  
again pressed into a container with saline and the suspen-  
sion is stirred with an aerating stirrer. The shearing  
effect of the stirrer also helps to reduce the size of  
15 aggregates of spheres. The stirring is continued until no  
further traces of  $\text{CH}_2\text{Cl}_2$  are expelled. This operation  
should be performed in a properly ventilated facility.

(b) Removal of air bubbles and released protein.

20

On settling, the white suspension separates into a floating  
layer of spheres with air bubbles, while the majority of  
the protein spheres settles to the bottom. The clear middle-  
layer is removed, cold degassed saline is added and after  
25 short gentle stirring and settling, this procedure is re-  
peated. After several repetitions, the suspension becomes  
clear and all spheres settle to the bottom. Six liters of  
packed beads have been obtained in a single run by following  
this procedure.

30

(c) Screening of beads.

Spheres which are too large or too small are now removed  
by washing and screening, such as by wet screening. The  
35 oversized and undersized spheres may be dissolved and re-  
cycled. Typically, a screen-size fraction between 260 and  
320  $\mu\text{m}$  will be retained. The volume of the packed retained  
beads is approximately five liters. It is important to note

1 that at this point, if no crosslinkers or solification  
agents have been present in the protein, gas (fluid gaseous  
medium) or suspension liquid (fluid liquid medium), the  
spheres and all the protein residue in the pipes, pumps,  
5 etc. are easily dissolved in hot water. This aids greatly  
in housekeeping and particularly in cleaning the screens.  
Further, it is significant that the processing steps have  
involved no toxic chemicals other than volatile  $\text{CH}_2\text{Cl}_2$ .

10 6. Diffusion hardening of spheres.

To the suspension of screened spheres in saline, approxi-  
mately one liter of 25% aqueous glutaraldehyde solution is  
added as a crosslinking agent for hardening purposes. The  
15 suspension is gently stirred at room temperature for 16  
hours. Then, the temperature is raised through a tem-  
perature ramp during which only occasional stirring is  
needed. A typical ramp is 25°C for 16 hours, 70°C for  
8 hours and 100°C for 16 hours. This is followed by  
20 exhaustive washings with saline until all glutaraldehyde  
has been removed. As explained above, neutralization is  
effected with a dilute solution of the collagen.

After neutralization, the spheres may be subjected to a  
25 100°C temperature for about 16 hours and then washed again  
in saline. The spheres may then be steam sterilized in a  
conventional fashion. The successive heat-treatments  
tighten the protein structure and render a steam-sterilizable  
sphere. At the same time the sphere dimensions contract  
30 to about 60% of original size resulting in a volume con-  
traction by a factor of five. By following the procedure  
described in this example, one liter of amber colored  
spheres of 150 to 190  $\mu\text{m}$  diameter have been obtained.

35 Use of 1, 4, butanediol diglycidyl ether or formaldehyde  
as a crosslinking agent in the procedures described herein  
has yielded colorless spheres. Addition of the reducing  
agent sodium dithionite to the glutaraldehyde crosslinking

1 agent has produced spheres which are nearly colorless.  
However, tests regarding the steam sterilizability of the  
spheres referred to in this paragraph have not been com-  
pleted.

5

#### 7. Use Of Cell Culture Microcarriers To Grow Diploid Human Foreskin Fibroblast Cells

Original cell stock of diploid human fibroblast cells

10 FS-4 is obtained from a roller bottle culture. The cells  
are removed from the bottle walls by trypsin treatment.  
To obtain a starter suspension, the content of the bottles  
is diluted with Dulbecco's modified Eagle's medium plus  
5% v/v foetal bovine serum to obtain a cell concentration  
15 of  $4 \times 10^5 \text{ ml}^{-1}$ .

To 100 ml of the starter suspension, 10 ml of packed cell  
culture microcarrier beads are added. The cells and beads  
(microcarriers) are stirred at 37°C in 10% CO<sub>2</sub>/90% air  
20 atmosphere. The attached cells are counted daily. A  
typical growth protocol is as follows:

	Day 0	.....	$4 \times 10^5$	cells per ml (starter suspension)
	Day 1	.....	$5,6 \times 10^5$	cells per ml.
25	Day 2	.....	$11 \times 10^5$	cells per ml.
	Day 3	.....	$11 \times 10^5$	cells per ml.

On the third day, the microcarrier culture is split for  
subcultivation. The microcarriers are allowed to settle  
30 and the supernatant is decanted. Beads are washed with  
Eagle's balanced salt solution, each time mixing the beads  
briefly with one half the original culture volume of  
solution, allowing the beads to settle and decanting the  
supernatant.

35

After washing, the beads are mixed with an equal volume  
of prewarmed (37°C) trypsin-EDTA solution to initiate cell  
detachment. The suspension is gently stirred for 10

1 minutes, at which point all cells have detached. One half  
of the bead volume of fetal bovine serum is then added  
and the microcarriers and cells are centrifuged at 250 G for  
ten minutes. The supernatant is discarded. To the cells  
5 and microcarriers, 20 ml of new cell culture microcarriers  
are added and the suspension is distributed into three  
spinner vessels bringing the content of the vessels to  
approximately 100 ml gross medium. A typical growth  
protocol for the subcultures is as follows:

10

Day 3 .....  $3.6 \times 10^5$  cells per ml (first day of split)  
Day 4 .....  $5.3 \times 10^5$  cells per ml  
Day 5 .....  $11 \times 10^5$  cells per ml.

15

20

25

30

35

1 CLAIMS:

1. A cell culture microcarrier comprising a water-insolubilized protein, said protein being substantially homogeneously distributed throughout said microcarrier.  
5
2. The microcarrier of claim 1, wherein said protein is selected from the group consisting of collagen, casein, soybean protein, silk, keratin, egg proteins and blood  
10 proteins.
3. The microcarrier of claim 2, wherein said protein is collagen.
- 15 4. The microcarrier of claim 1, wherein said microcarrier comprises the reaction product of said protein and a cross-linking agent.
5. The microcarrier of claim 4, wherein said crosslinking  
20 agent is multifunctional.
6. The microcarrier of claim 5, wherein said multifunctional crosslinking agent contains a functional group selected from the group consisting of aldehydes, epoxies,  
25 haloalkyls, isothiocyanates, isocyanates, diazo, arylnitrenes, azides, acylhalides, acylanhydrides, diolcarbonates, chloroformates, imidoesters, N-hydroxysuccinimidylesters, maleinimides, carboxydiimidazols, formylesters and halotriazines.  
30
7. The microcarrier of claim 6, wherein said multifunctional crosslinking agent is glutaraldehyde.
8. The microcarrier of claim 6, wherein said multifunctional crosslinking agent is 1, 4, butanediol diglycidyl  
35 ether.



- 1 9. The microcarrier of claim 4, wherein said crosslinking agent is selected for the group consisting of formaldehyde, epichlorohydrin, phosgene and aldehyde starch.
- 5 10. The microcarrier of claim 1, wherein said microcarrier has a diameter of about 50 to about 350 microns.
11. A method for producing cell culture microcarriers comprising the steps of:
- 10 (a) forming droplets of an aqueous solution of a protein;
- (b) suspending said droplets in a fluid;
- 15 (c) solidifying said suspended droplets into cell culture microcarriers; and
- (d) recovering said cell culture microcarriers.
- 20 12. The method of claim 11, wherein said protein is selected from the group consisting of collagen, casein, soybean protein, silk, keratin, egg proteins and blood proteins.
13. The method of claim 12, wherein said protein is
- 25 collagen.
14. The method of claim 11, wherein said droplets are formed by atomizing said solution from a pressure nozzle.
- 30 15. The method of claim 11, wherein said droplets are formed by atomizing said solution from a gas-propelled spray nozzle.
16. The method of claim 11, wherein said droplets are
- 35 formed by atomizing said solution from a rapidly spinning surface.
17. The method of claim 11, wherein said droplets are

1 formed by atomizing said solution from an ultrasonically  
driven nozzle.

18. The method of claim 11, wherein said droplets are  
5 formed by emulsifying said solution.

19. The method of claim 11, wherein said fluid contains  
a solidification agent for said droplets.

10 20. The method of claim 19, wherein said solidification  
agent is selected from the group consisting of ammonia,  
an aldehyde, an epoxy compound and epichlorohydrin.

21. The method of claim 20, wherein said aldehyde is a  
15 multifunctional aldehyde.

22. The method of claim 21, wherein said multifunctional  
aldehyde is glutaraldehyde.

20 23. The method of claim 20, wherein said epoxy compound  
is a multifunctional epoxy compound.

24. The method of claim 23, wherein said multifunctional  
epoxy compound is 1, 4 butanediol diglycidyl ether.

25  
25. The method of claim 20, wherein said aldehyde is  
formaldehyde.

26. The method of claim 11, wherein said fluid is a  
30 gaseous medium.

27. The method of claim 26, wherein said gaseous medium  
is air.

35 28. The method of claim 26, wherein said gaseous medium  
contains a compound selected from the group consisting of  
hydrogen chloride, ammonia, formaldehyde and glutaraldehyde.

- 1 29. The method of claim 11, wherein said fluid is a liquid medium.
30. The method of claim 29, wherein said liquid medium  
5 is immiscible with said droplets of said solution.
31. The method of claim 30, wherein said liquid medium is a halogenated hydrocarbon.
- 10 32. The method of claim 31, wherein said halogenated hydrocarbon is selected from the group consisting of methylene chloride, 1,1,1-trichloroethane, trichloroethylene and Freon<sup>(R)</sup> T.F.
- 15 33. The method of claim 29, wherein said liquid medium is miscible with said droplets.
34. The method of claim 33, wherein said liquid medium is an acid.
- 20 35. The method of claim 34, wherein said acid is an inorganic acid.
36. The method of claim 35, wherein said inorganic acid  
25 is selected from the group consisting of nitric acid, hydrochloric acid, phosphoric acid and sulfuric acid.
37. The method of claim 34, wherein said acid is an organic acid.
- 30 38. The method of claim 37, wherein said organic acid is selected from the group consisting of oxalic acid and formic acid.
- 35 39. The method of claim 33, wherein said liquid medium is a base.
40. The method of claim 39, wherein said base is selected

1 from the group consisting of ammonia, alkali hydroxide and  
an amine.

41. The method of claim 33, wherein said liquid medium  
5 is an aldehyde.

42. The method of claim 41, wherein said aldehyde is  
selected from the group consisting of formaldehyde and  
glutaraldehyde.

10

43. The method of claim 11, wherein said solidifying is  
accomplished by maintaining a temperature differential  
between said droplets of said solution of said protein  
and said fluid.

15

44. The method of claim 43, wherein said fluid is hotter  
than said droplets of said solution.

45. The method of claim 43, wherein said fluid is colder  
20 than said droplets of said solution.

46. The method of claim 43, wherein said temperature of  
said fluid is hotter than the temperature of said droplets  
of said solution and further, wherein said solution con-  
25 tains a solidification agent.

47. The method of claim 11, wherein said solidifying  
results from a solidification agent present in said so-  
lution of said protein.

30

48. The method of claim 47, wherein said agent is water  
soluble and is selected from the group consisting of  
aldehyde, acid and base.

35 49. The method of claim 11, further including, after the  
step of solidifying and prior to the step of recovering,  
the step of hardening.

- 1 50. The method of claim 49, wherein said hardening is  
accomplished by a crosslinking agent.
51. The method of claim 50, wherein the hardening effect  
5 of said crosslinking agent is accelerated by the appli-  
cation of heat.
52. The method of claim 51, wherein the hardening effect  
of said crosslinking agent is further accelerated by in-  
10 creasing the application of heat without liquifying said  
cell culture microcarriers.
53. The method of claim 52, wherein said protein is col-  
lagen, wherein said crosslinking agent is glutaraldehyde  
15 and wherein said heat is initially applied at 25°C for 1  
to 25 hours, followed by increasing said heat to 70°C for  
1 to 25 hours, followed by increasing said heat to 100°C  
for 1 to 25 hours.
- 20 54. The method of claim 52, wherein said heat is initially  
applied at 25°C for 15 to 20 hours, followed by applying  
said heat at 70°C for 6 to 10 hours, followed by applying  
said heat at 100°C for 15 to 20 hours.
- 25 55. The method of claim 50 wherein said crosslinking  
agent is multifunctional.
56. The method of claim 55, wherein said multifunctional  
crosslinking agent is selected from the group consisting  
30 of aldehyde and epoxy.
57. The method of claim 56, wherein said aldehyde is  
glutaraldehyde.
- 35 58. The method of claim 57, wherein said glutaraldehyde  
is combined with a reducing agent.
59. The method of claim 58, wherein said reducing agent

1 is sodium dithionite.

60. The method of claim 50, wherein said crosslinking agent is formaldehyde.

5

61. The method of any one of claims 19-25, 47-48 and 50-60, further including, prior to the step of recovering, the step of neutralizing excess solidification or cross-linking agent from said cell culture microcarrier.

10

62. The method of claim 61, wherein said neutralizing is effected by treating said microcarrier with a solution of said protein, said solution being sufficiently dilute to avoid solidification of said protein.

15

63. The method of claim 61, further including, after the step of neutralizing and prior to the step of recovering, the step of sterilizing said microcarriers.

20 64. The method of claim 61, further including, after the step of recovering, the step of sterilizing said microcarriers.

25 65. The method of any one of claims 11 - 18, 26 - 46 and 49, further including, prior to the step of recovering, the step of sterilizing said microcarriers.

30 66. The method of any one of claims 11-18, 26-46 and 49, further including, after the step of recovering, the step of sterilizing said microcarriers.

35 67. The method of claim 11, wherein said droplets are formed in a gaseous medium and wherein said fluid is a liquid medium.

68. The method of claim 67, wherein said liquid medium is immiscible with said droplets.

1 69. A method of growing anchorage-dependent cells comprising the steps of

(a) providing a suspension comprising cell culture  
5 microcarriers, an inoculum of said cells and a nutrient-containing growth medium, said microcarriers comprising a water-insolubilized protein, said protein being substantially homogeneously distributed throughout said microcarrier and

10

(b) maintaining said suspension under conditions conducive to cell growth.

70. A method of producing anchorage-dependent cells comprising the steps of

(a) providing a suspension comprising cell culture  
microcarriers, an inoculum of said cells and a nutrient-containing growth medium, said cell microcarriers comprising  
20 a water-insolubilized protein, said protein being substantially homogeneously distributed throughout said microcarrier,

(b) maintaining said suspension under conditions conducive to cell growth, and

(c) harvesting said cells.

71. A method of producing anchorage-dependent cell  
30 products comprising the steps of:

(a) forming a suspension, in a suitable cell culture medium, of cell culture microcarriers comprising a water-insolubilized protein, said protein being substantially  
35 homogeneously distributed throughout said microcarrier;

(b) inoculating said culture with anchorage-dependent cells to form a cell culture;

1 (c) maintaining said cell culture under conditions  
conducive to production of cell products; and

(d) harvesting said cell products.

5

72. The method of claim 71, wherein said cell growth  
product is interferon.

73. The method of any one of claims 69, 70 or 71, further  
10 including, immediately after the step of maintaining said  
suspension under conditions conducive to cell growth, the  
step of subcultivating said cell growth.

74. The method of claim 73, wherein said subcultivating  
15 is accomplished by separating said cells from said micro-  
carriers and introducing additional cell culture micro-  
carriers into said suspension

75. The method of claim 73, wherein said subcultivating  
20 is accomplished by separating said cells from said micro-  
carriers, removing said cells from said medium and said  
microcarrier, introducing said cells into a new medium and  
adding new cell culture microcarriers to said new medium  
containing said cells.

25

76. Apparatus for converting a water-based protein  
solution to microspheres comprising:

a heated, thermostatically controlled reservoir for  
30 the protein solution;

at least one spray head;

means for supplying the protein solution from the  
35 reservoir to said spray head;

means for applying heat to said supplying means for  
maintaining the protein solution in said at least one



1 spray head at approximately at least the same temperature  
as the protein solution in the reservoir;

means for maintaining a predetermined pressure on the  
5 protein solution at the spray head;

at least one receptacle positioned beneath the said  
spray head for receiving the droplets emitted by the spray  
head;

10

means for flowing a low temperature, water immiscible  
suspension liquid across said at least one receptacle for  
suspending and solidifying said received droplets into  
discrete microspheres in said suspension liquid; and

15

means for collecting said microspheres from said  
suspension liquid.

77. The apparatus of claim 76 wherein said supplying  
20 means includes a loop from and back to said reservoir said  
loop including an elongated, substantially horizontal pipe,  
said apparatus includes a plurality of spray heads spaced  
along said elongated, horizontal pipe and a like plurality  
of receptacles for individually receiving the droplets  
25 emitted by the spray heads and means for maintaining pre-  
determined temperatures throughout said loop.

78. The apparatus of claim 77, wherein said heated re-  
servoir includes a first section for containing the water-  
30 based protein solution and a second section for containing  
water for priming and flushing said circulating means and  
said spray heads.

79. The apparatus of claim 78, wherein said heated re-  
35 servoir includes means for maintaining both said first  
and second sections at a temperature of approximately  
50°C.

- 1 80. The apparatus of claim 77, wherein said supplying means includes a piston pump for forcing said protein solution through said loop and a buffer and restrictor in said loop for attenuating pulses created by said pump.
- 5 81. The apparatus of claim 78, wherein said loop includes a flexible member on the input of said loop and a flexible member on the output of said loop, wherein said flexible member may selectively be inserted either into said first  
10 section for circulating said protein solution or into said second section for circulating said heated water.
82. The apparatus of claim 81 also including a drain and wherein either or both of said flexible members may  
15 selectively discharge into said drain.
83. The apparatus of claim 81 including a screen in said first section for receiving the input end of said flexible member on the input of said loop.
- 20 84. The apparatus of claim 77, wherein each of said spray heads is dimensioned for discharging as atomized solution only a small fraction of the protein solution circulating through said loop.
- 25 85. The apparatus of claim 84, wherein each of said spray heads includes means for imparting a swirl to the jet emanating from the spray head.
- 30 86. The apparatus of claim 84, wherein each of said spray heads includes means for monitoring the temperature of the spray head.
- 35 87. The apparatus of claim 84, wherein each of said spray heads includes a noncircular elongated orifice.
88. The apparatus of claim 77, wherein said heat-applying means includes heating jackets selectively placed on said

1 loop.

89. The apparatus of claim 88 including heating jackets on the upstream sides of said spray heads.

5

90. The apparatus of claim 77, wherein said pressure maintaining means includes an adjustable throttle for adjusting the pressure of said protein solution on said spray heads.

10 91. The apparatus of claim 90 including a pressure gauge in said loop.

92. The apparatus of claim 77, wherein said flowing means includes means for circulating said suspension liquid  
15 through a loop.

93. The apparatus of claim 92, wherein said suspension liquid circulating means includes a reservoir and a pump.

20 94. The apparatus of claim 92 also including means for maintaining said low temperature suspension liquid at a temperature of approximately 0° to 10°C but not below the freezing point of the protein solution.

25 95. The apparatus of any one of claims 77, 92, 93 and 94, wherein each of said receptacles includes means for developing a pressure head of suspension liquid across an input section of each receptacle for increasing the speed of flow of the suspension liquid across the receptacle.

30

96. The apparatus of claim 95, wherein each of said receptacles is substantially rectangular, said pressure head developing means is a barrier across the input end of the receptacle and spaced slightly above the bottom of  
35 the receptacle and each receptacle includes a depressed area at the end opposite said barrier for producing a cascade across the rectangular receptacle.

- 1 97. The apparatus of claim 95, wherein said receptacles  
are dimensioned and positioned for receiving the droplets  
emanating from the individual spray heads only in the flow  
of the suspension liquid.
- 5 98. The apparatus of claim 77 also including closable  
gate means for selectively preventing entrance of the  
discharge from said spray heads into said receptacles.
- 10 99. The apparatus of claim 77, wherein said suspension  
liquid is a halogenated hydrocarbon.
100. The apparatus of claim 99, wherein said halogenated  
hydrocarbon is selected from the group consisting of  $\text{CH}_2$   
15  $\text{CL}_2$ , 1-1-1 trichloroethane, trichloroethylene and Freon<sup>®</sup><sub>2</sub>  
TF.
101. The apparatus of claim 77, wherein said collecting  
means includes means for separating said spheres from  
20 said suspension liquid.
102. The apparatus of claim 96 also including a common  
discharge for said receptacles and wherein said collecting  
means includes a filter in said common discharge.
- 25 103. The apparatus of claim 102 wherein said filter is a  
screen having a mesh size of about 500 um.
104. A method for converting a water-based protein solution  
30 into microspheres comprising the steps of:
- spraying the protein solution as a liquid into a  
gaseous atmosphere to form discrete droplets;
- 35 maintaining a temperature differential between the  
solution and the gaseous atmosphere wherein the gaseous  
atmosphere is at a lower temperature than the temperature  
of the solution before spraying;

1       flowing a cooled water-immiscible suspension liquid  
across the path of fall of said discrete droplets to form  
microspheres suspended in said cooled suspension liquid;  
and

5

collecting said cell culture microcarriers from said  
suspension liquid.

105. The method of claim 104, wherein the step of spraying  
10 includes the step of circulating an excess supply of  
protein solution across a plurality of spray heads.

106. The method of claim 105, wherein the step of cir-  
culating a supply of protein solution includes the step  
15 of maintaining the circulating protein solution at a  
temperature of about 50°C.

107. The method of claim 104, wherein said gaseous atmos-  
phere is air.  
20

108. The method of claim 104, wherein the step of flowing  
the cooled suspension liquid includes the step of main-  
taining the temperature of the suspension liquid slightly  
above the freezing temperature of the microspheres.  
25

109. The method of claim 105, wherein the step of flowing  
includes the step of developing individual flow paths across  
the fall from the plurality of spray heads.

30 110. The method of claim 109 including the step of  
dimensioning the spray cones from the spray heads and the  
individual flow paths for receiving the spray cones in-  
dividually and directly onto the flow paths.

35 111. The method of claim 109, wherein the step of de-  
veloping individual flow paths includes the step of forming  
a cascade in each flow path for receiving each spray cone  
in a cascade.

1 112. The method of claim 111, wherein the step of forming  
a cascade includes the steps of raising a pressure head  
in each flow path and depressing a portion of each flow  
path downstream from the pressure head.

5

113. The method of claim 109, wherein the step of collec-  
ting the spheres includes the steps of combining the in-  
dividual flow paths and separating the spheres from the  
combined flow path.

10

15

20

25

30

35

FIG. 1.

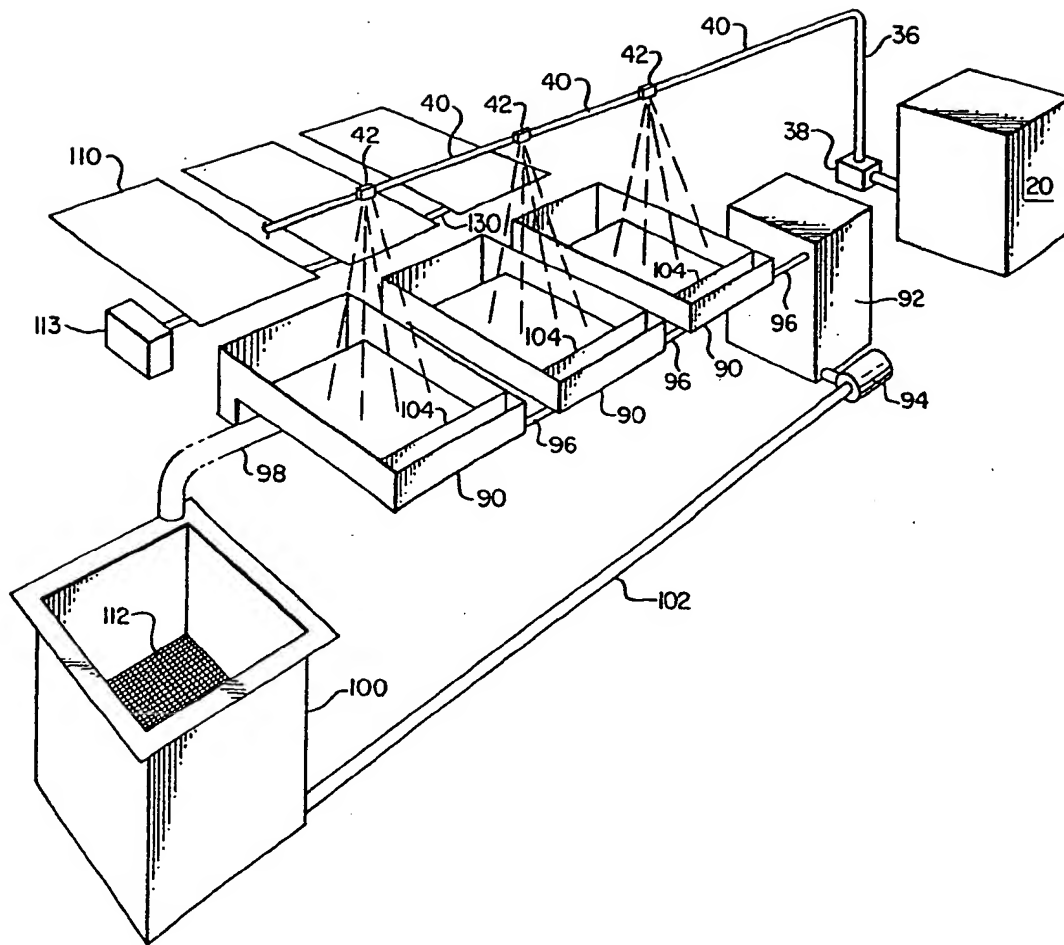


FIG. 2.

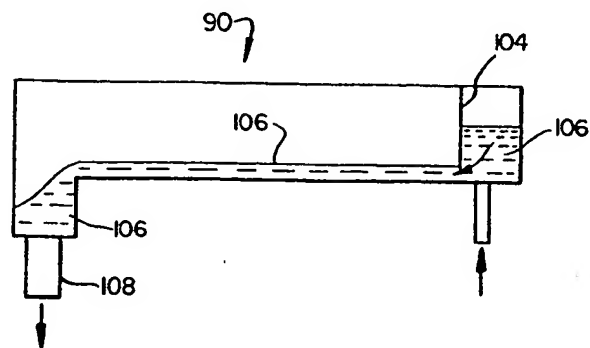


FIG. 3.

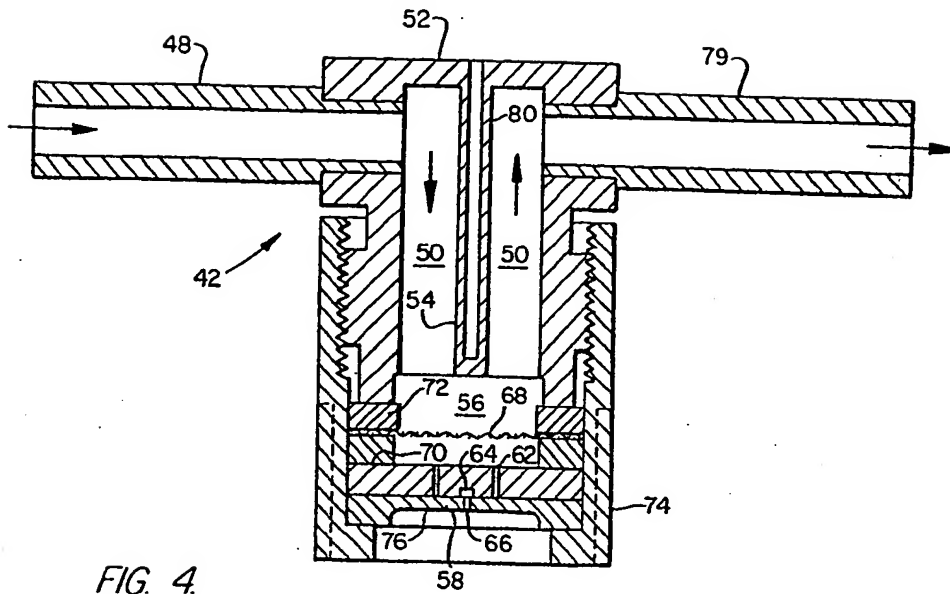


FIG. 4.

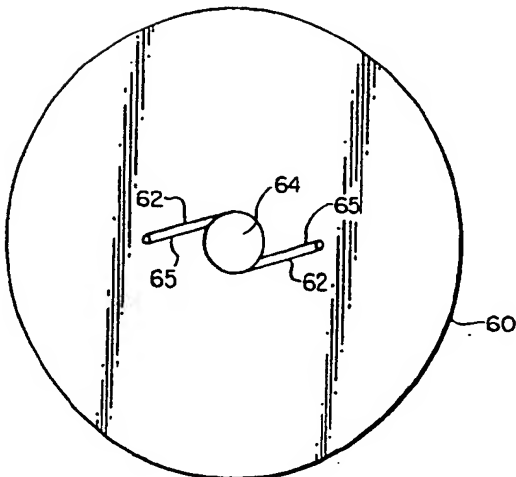


FIG. 5.

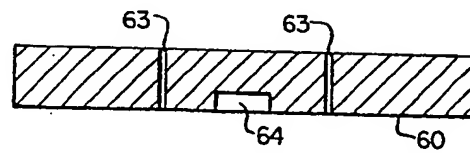


FIG. 8.

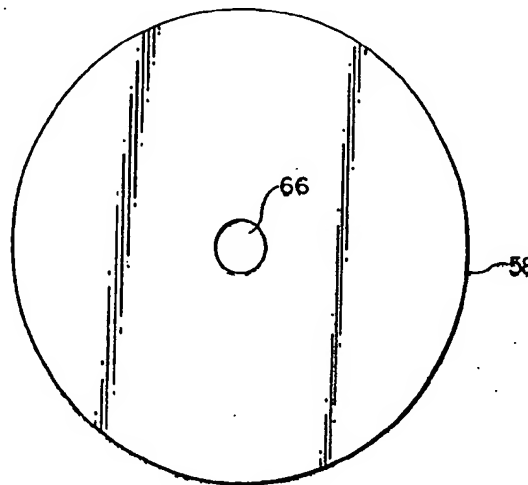
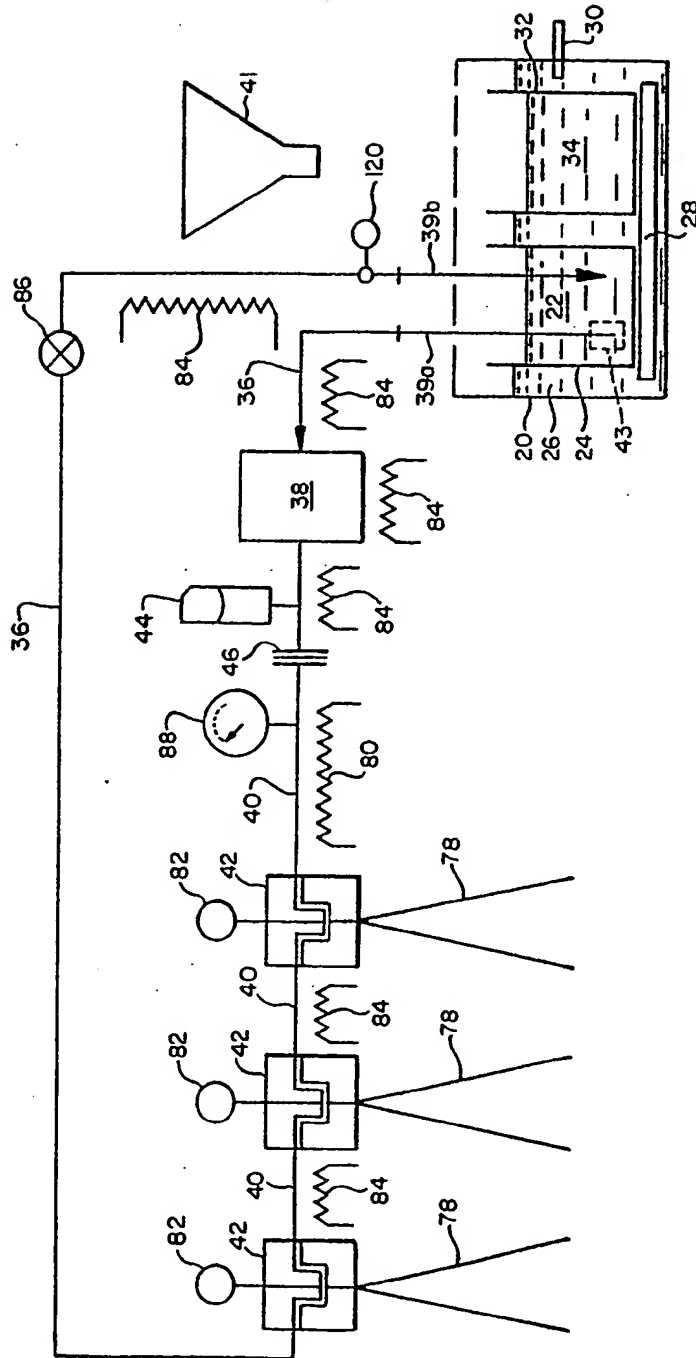




FIG. 6.



The flowchart illustrates the production of microcarriers, starting with the preparation of collagen solution and the main production cycle.

**Collagen Solution Preparation:** Water and collagen are combined and stirred hot. The resulting collagen solution is filtered and used for make-up.

**Main Production Cycle:**

- Beads in Saline:** The process begins with beads in saline, which are aerated and sheared. Air and  $\text{CH}_2\text{Cl}_2$  are added.
- Mashed Cake:** The beads are mashed in saline, with air and saline added.
- Strained:** The mixture is strained, separating the bead cake from the collagen bead suspension in  $\text{CH}_2\text{Cl}_2$ .
- Impactation + Cooling:** The bead cake is subjected to impactation and cooling, with air added.
- Collagen Aerosol:** The collagen bead suspension is sprayed, cooled, and forms a collagen aerosol.
- Washing + Screening:** The aerosol is washed and screened to produce screened beads.
- Recycled Collagen:** The  $\text{CH}_2\text{Cl}_2$  phase is recycled back into the process.
- Exhaust:** Air is exhausted from the system.

**Final Processing:**

- Micro-carrier:** The screened beads are sterilized.
- Wash:** The micro-carriers are washed with saline.
- Stirr:** The micro-carriers are stirred.
- Dilute Collagen:** Dilute collagen is added to the stirred micro-carriers.
- Wash:** The mixture is washed with saline.
- Treat-Waste:** The waste is treated.
- Reducer:** The micro-carriers are reduced.
- Glut. Aldeh.:** The micro-carriers are treated with glutaraldehyde.
- Temperatures:** The process involves temperatures of 100°C, 70°C, and 25°C.

**EUROPEAN PATENT APPLICATION**

Application number: 83106050.4

Int. Cl.: **B 01 J 13/00, C 12 N 5/02,**  
**C 12 P 21/02, B 01 J 2/02**

Date of filing: 21.06.83

Priority: 25.06.82 US 392218

Applicant: **FLOW GENERAL, INC.**, 7655 Old Springhouse Road, Mclean Virginia 22102 (US)

Date of publication of application: 11.01.84  
Bulletin 84/2

Inventor: **Haller, Wolfgang K.**, 4620 N. Park Avenue Apt. 909E, Chevy Chase, MD (US)

Designated Contracting States: **BE DE FR GB IT NL SE**

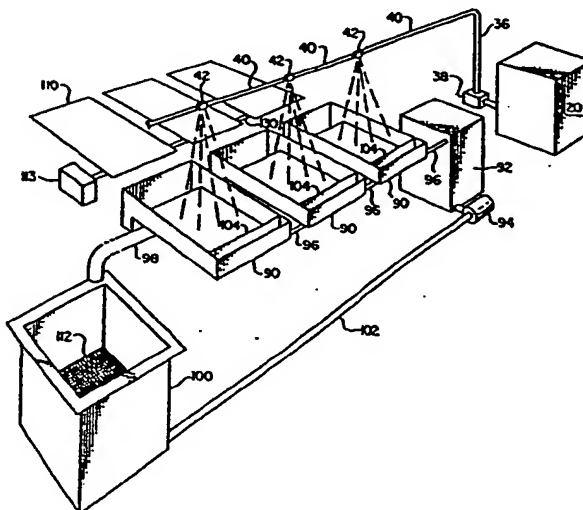
Date of deferred publication of search report: 09.01.85 Bulletin 85/2

Representative: **Patentanwälte Grünecker, Dr. Kinkeldey, Dr. Stockmair, Dr. Schumann, Jakob, Dr. Bezold, Meister, Hilgers, Dr. Meyer-Plath,** Maximilianstrasse 58, D-8000 München 22 (DE)

**Cell culture microcarriers.**

Cell culture microcarriers made of water-insolubilized protein. The protein is substantially homogeneously distributed throughout the microcarrier. The cell culture microcarrier is made by forming droplets of an aqueous solution of a protein, suspending the droplets in a fluid, solidifying the suspended droplets into cell culture microcarriers and recovering the cell culture microcarriers. Preferably, prior to recovery, the cell culture microcarriers are hardened with a cross-linking agent, preferably glutaraldehyde, and neutralized with a dilute solution of the protein. An apparatus for converting a water-based protein solution to microspheres. The apparatus has a heated, thermostatically controlled reservoir (20) for the protein solution. The protein solution is supplied to a spray head (42). The protein solution is maintained at approximately at least the same temperature at the spray head as in the reservoir, and a predetermined pressure is maintained on the protein solution at the spray head. A receptacle (90) receives the droplets emitted by the spray head. The droplets are suspended and solidified into microspheres by flowing a low temperature, water immiscible suspension liquid across the receptacle, and the microspheres are collected. The cell culture microcarriers have been used to grow and harvest anchorage dependent cells and anchorage dependent cell products, such as interferon. The cell culture microcarriers

produce outstanding cell growth and possess superior sub-cultivation ability.





DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 3)
X	EP-A-0 030 885 (INSTITUT PASTEUR)  * Page 2, line 16 - page 4, line 23; page 5, lines 13-30 *	1-7, 10 -22, 29 , 30, 50 , 60, 61 , 69-71	B 01 J 13/00 C 12 N 5/02 C 12 P 21/02 B 01 J 2/02
X	WO-A-8 200 660 (CORNING GLASS)  * Page 10, lines 6-31 *	1-7, 29 , 30	
A	GB-A-2 059 991 (PHARMACIA FINE) * Page 2, lines 19-25 *	1	
A	EP-A-0 041 344 (TORAY INDUSTRIES) * Abstract *	1, 72	
			TECHNICAL FIELDS SEARCHED (Int. Cl. 3)
			C 12 N C 12 M B 01 J
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 02-10-1984	Examiner DAUKSCH H. J.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons  & : member of the same patent family, corresponding document	